PB81-232050

Isolation of Mutants Temperature-Sensitive for Expression of the Transformed State from Chemically Transformed C3H/10T1/2 Cells

University of Southern California
Los Angeles

Prepared for

Health Effects Research Lab.
Research Triangle Park, NC

14 Nov 80

U.S. Department of Commerce
National Technical Information Service

TECHNICAL REPORT DATA

(Please read Instructions on the reverse before completing)

1. REPORT NO. EPA-600/J-80-307	2. JOURNAL ARTICLE	3. PB81-232050
4. TITLE AND SUBTITLE: Isolation of mutants temperature-sensitive for expression of the transformed state from chemically transformed C3H/10T1/2 cells		5. REPORT DATE: July 1981 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S): Craig Borelko and Charles Heidelberger		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS: University of Southern California, Comprehensive Cancer Center, 1303 North Mission Road, Los Angeles, CA 90033		10. PROGRAM ELEMENT NO. C9XA1C 11. CONTRACT/GRANT NO. Grant R-805208
12. SPONSORING AGENCY NAME AND ADDRESS: Office of Research and Development (RTP,NC), Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

Published In: Carcinogenesis 1(12): 1059-1073, 1980

16. ABSTRACT

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17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

PRODUCT OF:
NATIONAL TECHNICAL INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	
	20. SECURITY CLASS (This page) UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (9-73)

Carcinogenesis, Volume 1 Number 12 1980

EPA-600/J-80-307
Journal Article

Isolation of mutants temperature-sensitive for expression of the transformed state from chemically transformed C3H/10Tl/2 cells

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(Received on 6 October 1980; accepted on 14 November 1980)

Abstract

58 MCA Cl 16 is an oncogenic methylcholanthrene-transformed variant of the non-transformed mouse embryo fibroblast cell line, C3H/10T1/2 Cl 8. Using two different protocols, we have isolated six temperature-sensitive mutants from N-methyl-N′-nitro-N-nitrosoguanidine treated cultures of 58 MCA Cl 16. C3H/10T1/2 Cl 8, 58 MCA Cl 16 and the six mutant lines were characterized with respect to several properties associated with the transformed state: morphology, saturation density, anchorage independence, cell surface morphology and growth in medium containing 1% fetal calf serum. In general, C3H/10T1/2 cells behaved as non-transformed, whether grown at 33°C or 39.5°C. The transformed parental line and all six mutants behaved as transformed cells at 33°C. At 39.5°C, only the parental transformed line retained the transformed phenotype. Three of the mutants revert towards non-transformed behavior at 39.5°C for all of the properties tested. The remaining mutants are temperature-sensitive for some, but not all, transformed characteristics. Thus, while the expression of these transformed properties is sometimes coupled, we have been able to dissociate the expression of traits such as saturation density, anchorage independence and transformed morphology from each other. These mutants should prove to be valuable tools in the study of the mechanisms which underly the expression of the chemically-induced transformed state.

Introduction

The non-tumorigenic mouse embryo fibroblast cell line C3H/10T1/2 Cl 8 (1) has gained widespread use as a system for the study of oncogenic transformation by chemical and physical agents (for a review see ref. 2). Relatively little is currently known of the precise molecular changes that underly the process of oncogenic transformation by chemicals (3,4). In contrast, the gene products primarily responsible for transformation by the RNA tumor viruses (5) and papova viruses (6-8) have been isolated, leading to advances in our understanding of their modes of action.

Many of the significant advances in the field of viral oncogenesis have been made possible by the study of temperature-sensitive (ts)* mutants of viruses that are able to establish and/or maintain the transformed state at one temperature but not at another. Such mutants provide excellent model systems for the study of oncogenesis, and permit the comparison of the transformed and non-transformed states in cells that are genotypically identical. Ts mutants also provide a means by which markers can be introduced into gene sequences associated with transformation, thus facilitating genetic analysis (for reviews see refs. 8,9).

While ts mutants of mammalian cells are now well known (10), few cellular mutants affecting expression of the transformed state have been isolated. The first of such ts mutants was reported by Renger and Basilico (11), who employed multi-step selection procedures to isolate apparent cellular mutants affecting the expression of transformation in SV40 transformed 3T3 cells. While these mutants contained wild-type SV40 genomes and synthesized viral T antigens independently of the temperature at which the cells were grown, the transformed state was only expressed at 32°C. When grown at 39°C, the cells behaved as non-transformed 3T3 cells for most properties tested.

Several investigators have since established the feasibility of isolating cells that exhibit ts modulation of the spontaneous and chemically-induced transformed state. Miyashita and Kakunaga (12) have selected both heat- and cold-sensitive mutants from mutagenized cultures of spontaneously transformed Chinese hamster lung cells. Curiously, ts cells were found to arise spontaneously in BALB/3T3 cultures (13,14). Unfortunately, details of the isolation of these cells are scanty, their stability in at least one instance is questionable, and evaluation of their phenotypes is made difficult by unspecified viability changes associated with their ts behavior. Similarly, a high passage C3H mouse cell line, C3H2K, has been reported to exhibit a transformed morphology and increased saturation density at 33°C but not at 38°C

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*Abbreviations: FUdR, 5-fluoro-2′-deoxyuridine; MNNG, N-methyl-N′-nitro-N-nitrosoguanidine; BME, basal medium Eagles; ts, temperature-sensitive; FCS, fetal calf serum.

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(15). The significance of this behavior is unknown, and its relationship to oncogenicity is made uncertain by the failure of the cells to exhibit anchorage independence at either temperature.

Ts lesions affecting the chemically transformed state have been isolated in two different cell culture systems. Di Mayorca and colleagues (16-18) have reported that a high percentage of chemically transformed BHK 21 cells are ts for the expression of transformation. That ts behavior should be found in a large proportion of transformed clones is surprising, and it has been suggested (10) that this may be indicative of selection pressures inadvertently applied during transformation. The low frequency with which such transformants are recovered (6×10^{-6}) supports this suggestion and raises the possibility that the mutants isolated by di Mayorca represent a limited class of transformants.

Yamaguchi and Weinstein (19) have succeeded in isolating ts mutants of a chemically transformed rat liver epithelial cell and have since used these mutants in the study of the role played by glycopeptide metabolism in transformation (20). Interpretation of their results is difficult, however, since the phenotypic characteristics that accompany epithelial cell transformation have yet to be clearly defined. Their mutants were determined to be ts for transformation primarily on the basis of assays for plating efficiency in soft agar. In addition, their mutant and parental lines are known to contain an unidentified transforming virus. While this virus is not ts in the mutants, one cannot exclude the possibility that the virus is involved in the expression of the chemically-induced transformed state. The ts behavior of these mutants might thus reflect alterations in cellular mechanisms that govern expression of this virus.

Convinced of the need to develop ts model systems more appropriate to the study of chemical carcinogenesis, we attempted to isolate ts mutants from chemically transformed C3H/10T1/2 cells. These cells are transformed by a variety of chemical and physical agents without any demonstrable mediation by retroviruses (21,22). We report here the isolation and preliminary characterization of six ts mutant cell lines. All mutants are fully viable at both permissive and non-permissive temperatures and have been examined with respect to several properties considered relevant to the oncogenic transformation of fibroblasts.

Materials and Methods

Cell culture

Mutants were obtained from the cell line 58 MCA Cl 16 (23), an oncogenic transformed variant isolated from methylcholanthrene-treated C3H/10Tl/2 Cl 8 cells. 58MCA Cl 16 cells were used between passages 29 and 36. Other studies were conducted using C3H/10T1/2 Cl 8 cells (1) between passages 5 and 12.

Procedures for the routine handling of cell lines have been described previously (1). Briefly, cells were cultured in basal medium Eagle's (BME) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco). For the purposes of the studies described here, stock cultures of all cell lines used were maintained at 33°C in humidified incubators with an atmosphere of 5% CO_2. The cells were regularly subcultured at confluence (approximately every 10 days) in 25 cm^2 plastic flasks (Corning) at initial seeding densities of 5×10^4 cells per flask.

Saturation densities and doubling times were determined by growth curve analysis. For each cell line, 5×10^3 cells were plated in 60 mm dishes (Corning) in 5 ml of medium, which was changed twice weekly. Every 24 h, or longer if growth was slow, two dishes were randomly selected, washed with 0.9% saline, treated with 0.1% trypsin (Gibco) and the total cell number in each dish counted with a Model B Coulter Counter. Values for each day were averaged, plotted on a graph of log cell numbers *vs* time, and doubling times were determined by linear regression analysis of the logarithmic portion of the curves. For cell lines exhibiting density-dependent inhibition of cell growth, saturation densities were calculated as the average of the total cell number present at confluence for at least three separate sampling times after confluence was reached. In growth curves where density-dependent inhibition of growth was not evident, saturation densities were arbitrarily designated as the highest cell number per dish reproducibly reached at least 12 days post-confluence. Doubling times in 1% FCS were determined by growth curve analysis following the preceding methods. For some cell lines, saturation densities were determined on dishes coated with poly-L-lysine by the procedure of McKechan and Ham (24). Non-sterile 0.5 ml aliquots of a 0.1 mg/ml solution of poly-L-lysine were spread over the surface of 60 mm dishes. After 10 min, the solution was removed, the dishes washed three times with 5 ml of sterile double-deionized water and exposed for 90 min to a germicidal lamp. The dishes were then seeded with 5×10^4 cells and treated by the same procedures used for growth curves.

Determinations of morphology were made in cultures confluent for 1–2 weeks. Some cultures were grown on poly-L-lysine treated dishes. Photographic records were taken of living cultures using Panatomic X film and a Model MSS Nikon binocular inverted microscope equipped with AFMB automatic

exposure photographic apparatus. Morphology was assessed using the criteria of Reznikoff *et al.* (23). Scoring was confirmed by examining cultures fixed in methanol and stained with 5% Giemsa.

To assay for anchorage independence, cells were plated in 2 ml overlays of 0.25% agarose onto previously prepared 5 ml base layers of 0.4% agarose in 60 mm dishes, following the basic procedures of Macpherson (25) for soft agar culture. All agarose layers contained the equivalent of single-strength BME supplemented with 10% FCS. Dishes were fed once weekly with 2 ml of complete medium, and colonies larger than 16 cells were scored microscopically after 3 weeks. In these assays, $5 \times 10^2 - 10^5$ cells were plated per dish.

Methyl cellulose culture was performed following the basic procedures of Stoker (26) as used by Vogel and Pollack (27). Cells were plated in 0 ml aliquots of a 1% methocel overlay onto 10 ml base layers of 0.9% agarose in 100 mm plastic dishes. All methocel and agarose layers contained the equivalent of single strength BME supplemented with 10% FCS.

Plating efficiencies on plastic dishes were determined by plating 100-200 cells in 60 ml dishes in 5 ml of complete medium. Following 10-14 days of incubation, the dishes were fixed in absolute methanol, stained with 5% Giemsa, and colonies scored using a dissecting microscope.

Scanning electron microscopy

Scanning electron microscopy of cell preparations was performed following a variation of the procedures of Porter *et al.* (28). Cells were seeded at low density into dishes containing 1 cm^2 glass coverslips and grown until about 25% confluent at the appropriate temperature. The coverslips were then fixed for 1–2 h at room temperature in Karnovsky's fixative and stored at 4°C in 0.1 M sodium cacodylate buffer containing 0.25 M sucrose. Samples were post-fixed for 2 h in 1% buffered osmium tetroxide, dehydrated through a graded series of ethanol solutions and critical point dried from liquid CO_2 using a Ladd critical point drier. Specimens were coated with a thin layer of 60:40 gold:palladium, and pictures taken using an AMR 1000 scanning electron microscope at 20 KV.

Chemicals

N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG) was obtained from Aldrich and dissolved in acetone just prior to use. MNNG treatment of cells was initiated by the addition of 0.03 ml of the MNNG: acetone solution per 15 ml of serum-free medium, and terminated with a medium change. 5-Fluoro-2′-deoxyuridine (FUdR) was obtained from Sigma Chemical Co., or was provided as a gift from Hoffman-LaRoche. Uridine and poly-L-lysine (Type 1-B) were purchased from Sigma. Prior to use, solutions of uridine and FUdR were prepared in phosphate-buffered saline, sterilized by filtration, and kept frozen until use.

Results

Isolation of ts mutants

Preliminary studies had demonstrated that C3H/10 T1/2 cells or their chemically transformed variants were not already ts for the expression of transformation (data not shown). Two protocols were employed to isolate different series of ts mutants from chemically transformed and oncogenic 58MCA Cl 16 cells. As will be discussed, "MTS" mutants were isolated following selection in methyl cellulose culture. Mutants of the "TSC" series were isolated by a modification of the confluence selection procedures of Renger and Basilico (11) as later employed by Yamaguchi and Weinstein (19). As shown in Figure 1, 1×10^6 logarithmically 58MCA Cl 16 cells, growing at 33°C in six different 100 mm plastic pertri dishes, were treated for 3 h with 0.3 μg/ml of MNNG. Preliminary work had shown that this level of MNNG constituted the LD50 concentration. The cells were allowed to recover at 33°C. As confluence was being reached, the cultures were provided with fresh medium and shifted to 39.5°C After 24 h of incubation at 39.5°C, to allow expression of any ts lesions, FUdR and uridine were added to the medium at a final concentration of 30 μg/ml of FUdR and 300 μg/ml of uridine. Following 72 h of exposure to FUdR/uridine, the cultures were washed four times with BME containing no serum; complete medium was then added, and the cultures were returned to 33°C. After 24 h incubation at 33°C, the cultures were trypsinized and replated at a 1:2 dilution in 100 mm dishes and grown again to near confluence. The

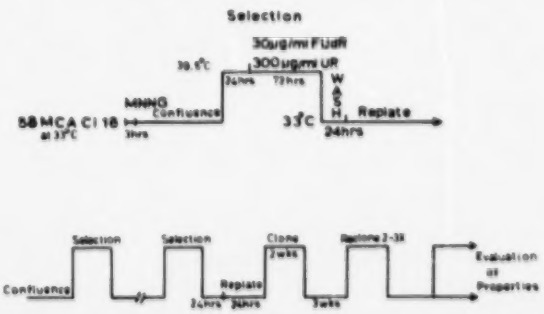

Fig. 1. Selection protocol for the isolation of ts mutants.

cycle of FUdR/uridine treatment was then repeated twice more.

Preliminary work had shown that the timing of medium changes and replatings was critical. Confluent C3H/10T1/2 cells were quiescent and protected from the toxic effects of FUdR if they were not provided with fresh medium for 24 h prior to FUdR treatment, if treatment lasted a maximum of 72 h, and if cultures were allowed 24 h to recover before replating. Under these conditions, 58MCA Cl 16 cultures experienced a 10–30% reduction in cell number during treatment, and only 30% of the remaining cells were viable as assayed by plating efficiency. More importantly, treated 58MCA Cl 16 cultures showed a protracted lag period of 5 days after replating before resuming growth. This cytotoxicity and lag period combined to give a potential 50-fold enrichment of treated cultures for the presence of putative ts mutants.

Following three cycles of treatment, the 58MCA Cl 16 cultures were replated at low density at 39.5°C and the dishes incubated until colonies had formed. Dishes were then microscopically examined and colonies exhibiting a flat, reverted morphology were noted. Approximately 1.5% of the 4500 colonies examined had such characteristics, and 41 of these were picked, using cloning rings, from six independently treated cultures. The majority of these colonies grew to form what appeared to be cultures of slowly growing transformed cells. Of 18 colonies seeming to exhibit ts differences in saturation density at 39.5°C and 33°C, six (one from each selection group) were recloned 2–3 times and their ts behavior examined with respect to morphology and saturation density. TSC-1, TSC-2,, and TSC-5 possessed the best ts phenotypes and were then examined in greater detail.

The MTS series of mutants were isolated in methyl cellulose following procedures similar to those of Miyashita and Kakunaga (12); the basic protocol was very similar to that depicted in Figure 1. 58MCA Cl 16 cells were grown at 37°C in 100 mm plastic petri dishes. When approximately 1 x 10^6 cells were present per dish, six dishes were treated with 0.3 μg/ml of MNNG as described previously. Following treatment, the cultures were allowed to recover at 33°C. When the dishes were confluent, they were shifted to 39.5°C for 24 h, trypsinized, and the contents of each dish plated into methyl cellulose suspension culture in the presence of 30 μg/ml of FUdR and 300 μg/ml of uridine. Approximately 5 x 10^5 cells were present per ml of overlay. After 72 h of incubation at 39.5°C, the cells were harvested from the methyl cellulose by the addition of 10 ml of cold (4°C) BME to each dish, refrigeration of each dish at 4°C for 1 h and pipetting of the liquified contents of each dish into 50 ml centrifuge tubes. The dishes were then washed with two 10 ml aliquots of cold MBE, and the washes were mixed with the liquified methyl cellulose. The cells were then centrifuged, resuspended and centrifuged three additional times to remove FUdR. The harvested cells were plated at a 1:2 dilution at 33°C, grown to confluence, and the cycle of treatment repeated twice more.

Preliminary experiments had revealed that non-transformed cells remain viable for 72 h when suspended in methylcellulose in the presence or absence of FUdR, but do not divide (data not shown). During this time, 58MCA Cl 16 cells under-

Table I

Effect of temperature on morphology, doubling time and saturation density.

Cell line	Morphology[a]		Doubling time (h)		Saturation density (cells/cm² x 10^{-4})		
	39.5°C	33°C	39.5°C	33°C	39.5°C	33°C	33°/39.5°C[c]
C3H/10T1/2	N	N	16.5 ± 0.5	22.1 ± 2.1	3.0	4.3	1.4
58MCA Cl 16	Tx	Tx	14.6 ± 0.7	23.0 ± 3.3	45.0	27.0	0.6
TSC-1	N	Tx	17.0 ± 0.2	23.0 ± 0.5	5.2	23.0	4.6
TSC-2	N	Tx	16.6 ± 2.2	27.4 ± 5.3	9.4	19.0	2.0
TSC-5	Tx	Tx	16.7 ± 2.0	27.9 ± 3.5	4.0	10.0	2.7
MTS-1	N	Tx	15.0 ± 0.1	25.2 ± 0.7	6.5	19.0	3.1
MTS-4	Tx	Tx	19.5 ± 0.1	30.8 ± 0.1	28.0	19.0	0.67
MTS-6	N	Tx	17.2 ± 1.8	26.6 ± 2.8	5.6	14.0	2.6

[a] N = non-transformed; Tx = transformed.

[b] Data for TSC-1, TSC-5, MTS-1 and MTS-6 were collected on poly-L-lysine treated dishes.

[c] Ratio of saturation density at 33°C: saturation density at 39.5°C.

Data are the mean of 2–4 experiments for each numerical entry.

went between one and three cell divisions. If FUdR were present, 98% of the cells lost viability. As found for FUdR treatment at confluence, the surviving cells had an increased lag phase upon replating. This lag phase was not as pronounced as was observed with confluence treatment, and resulted in about 20-fold potential enrichment for ts mutants per cycle.

After three rounds of such treatment, cells were plated at low density at 39.5°C and colonies examined as before. Only 1% of approximately 6000 colonies examined had a flat morphology and 40 of these were isolated. An additional 30 colonies of transformed morphology were also isolated. Of the 40 flat colonies isolated, only two were found to exhibit ts behaviour with respect to saturation density, and/or anchorage independence; the remainder were comprised of either slow growing transformed cells or non-ts revertants. The two ts lines were designated MTS-1 and MTS-6. Of the 30 colonies with transformed morphology isolated, only one was ts for anchorage independence and was designated MTS-4. These three lines were recloned 2–3 times, and examined in greater detail.

Effect of temperature on cell growth and morphology

The growth characteristics of 58MCA Cl 16, C3H/10Tl/2 Cl 8, and the six mutant clones were examined in plastic dishes at 39.5°C and 33°C. All clones had similar doubling times at each temperature (Table I); at 33°C they grew more slowly than at 39.5°C. Interesting differences became evident when saturation densities and morphologies were examined (Table I). Whether grown at 39.5°C or 33°C, 58MCA Cl 16 (Figure 2A-B) and MTS-4 exhibited a transformed morphology and grew to high cell density. Lower cell densities were generally observed at 33°C, due to the peeling of the cells from the dishes with medium changes. The confluent dishes were composed of multilayered sheets of polar cells growing in a random, criss-crossed fashion. In contrast, C3H/10Tl/2 Cl 8 cells at either temperature were morphologically non-transformed (Figure 2C-D), since confluent dishes were composed of monolayers of regularly spaced cuboidal cells displaying density-dependent inhibition of cell growth. Saturation densities at 33°C were a little higher than those at

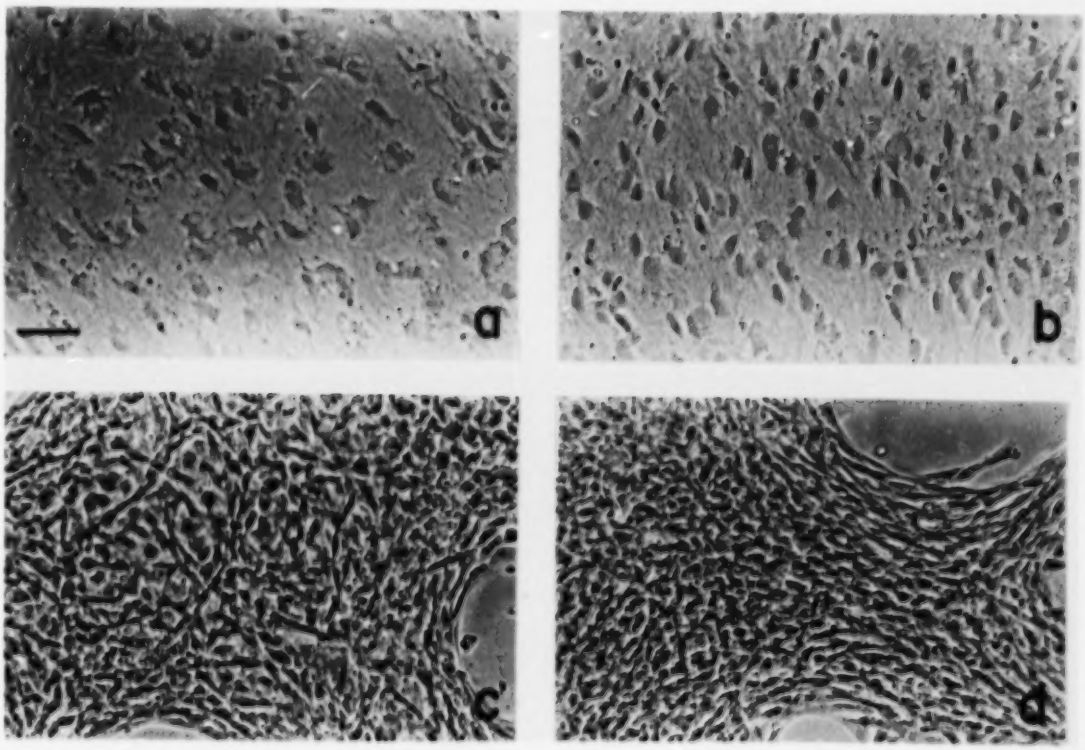

Fig. 2. Phase-contrast photomicrographs of wild-type cells at confluence. Bar = 30 μM. (A) 58 MCA Cl 16 at 39.5°C. (B) 58 MCA Cl 16 at 33°C. (C) C3H/10Tl/2 Cl 8 at 39.5°C. (D) C3H/10Tl/2 Cl 8 at 33°C.

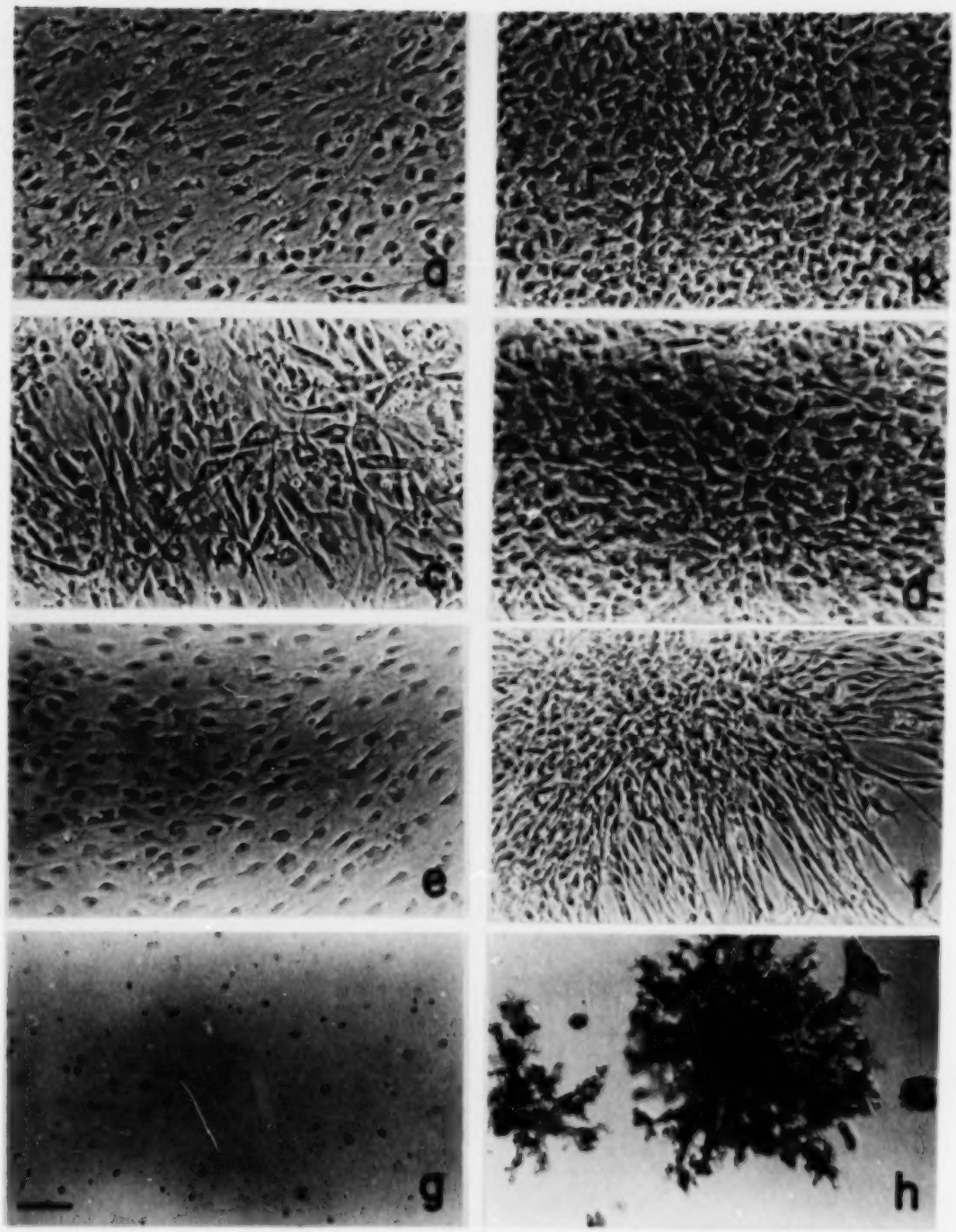

Fig. 3. Phase-contrast photomicrographs of confluent ts mutant cell lines grown on poly-L-lysine. Bar = 30 μM. (A) TSC-1 at 39.5°C. (B) TSC-1 at 33°C. (C) TSC-5 at 39.5°C. (D) TSC-5 at 33°C. (E) MTS-6 at 39.5°C. (F) MTS-6 at 33°C. Photomicrographs of colonies in soft agarose. Bar = 85 μM. (G) TSC-1 at 39.5°C. (H) TSC-1 at 33°C.

39.5°C, a reflection of monolayers in which the cells were spaced a little closer together.

At 33°C, all five of the remaining mutant clones grew to high cell densities and displayed the transformed morphology described above (although TSC-1 was not as polar as the rest). At 39.5°C, all five mutant lines grew to a definite saturation density somewhat higher than that of the non-transformed C3H/10T½ Cl 8 cells. For TSC-1, TSC-5 and MTS-6, these saturation densities were quite stable. TSC-2 and MTS-1 displayed a tendency to form heavier monolayers when supplied with fresh medium, perhaps reflecting leakiness of the ts leson. Four of the five lines also exhibited a non-transformed morphology at 39.5°C; the cells generally appeared to be cuboidal and regularly spaced at confluence. The exception to this was TSC-5, which exhibited density-dependent inhibition of cell growth at 39.5°C, and the cells at confluence appeared polar and criss-crossed. Representative photographs depicting these temperature-dependent effects on growth for three of the ts mutants are included in Figure 3.

The saturation density and morphology data collected for four of these mutant clones were performed on poly-L-lysine treated dishes (24). When grown on untreated dishes at 33°C, TSC-1, TSC-5, MTS-1 and MTS-6 displayed problems of attachment at confluence; the medium changes necessary to maintain viability in heavily confluent dishes caused cells to slough off in large sheets. Growing the cells on poly-L-lysine treated dishes enhanced attachment and permitted higher saturation densities to be reached at 33°C, but did not alter saturation density at 39.5°C. Morphology at either temperature also seemed to be unaffected by growth on poly-L-lysine. Estimates of growth rates for all lines were obtained using untreated dishes.

Effect of temperature shifts on growth

In order to examine whether or not the ts lesion in mutant cell lines was freely reversible, reciprocal temperature shift experiments were performed using confluent cultures. To do this, all cell lines were plated in 60 mm dishes and grown to confluence at either 33°C or 39.5°C. When dishes had been confluent for about 7 days, randomly selected dishes were shifted from 39.5°C to 33°C, or from 33°C to 39.5°C. Changes in cell number were then monitored.

These reciprocal temperature shift experiments (Figure 4 and 5) demonstrated that the lesion in cell lines ts for saturation density was freely reversible. In the shift from 33°C to 39.5°C, cell numbers began to decline 24 h or less after the shift, and dishes of multilayered transformed appearing cells formed flat

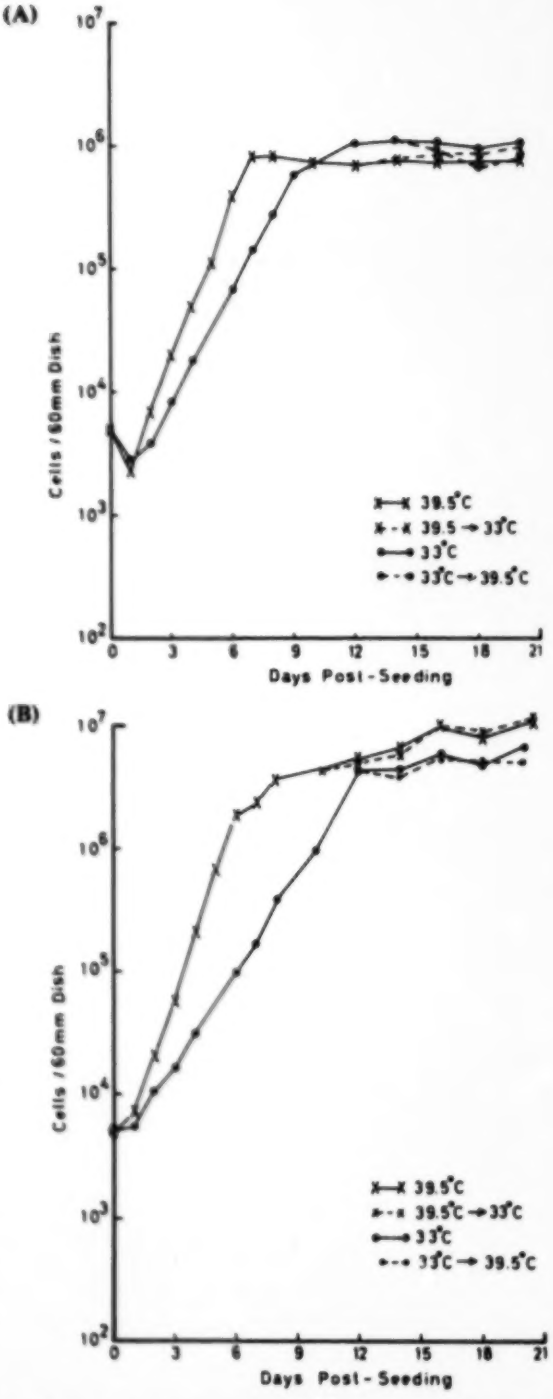

Fig. 4. Reciprocal temperature-shift growth curves for (A) C3H/10T½ Cl 8. (B) 58 MCA Cl 16.

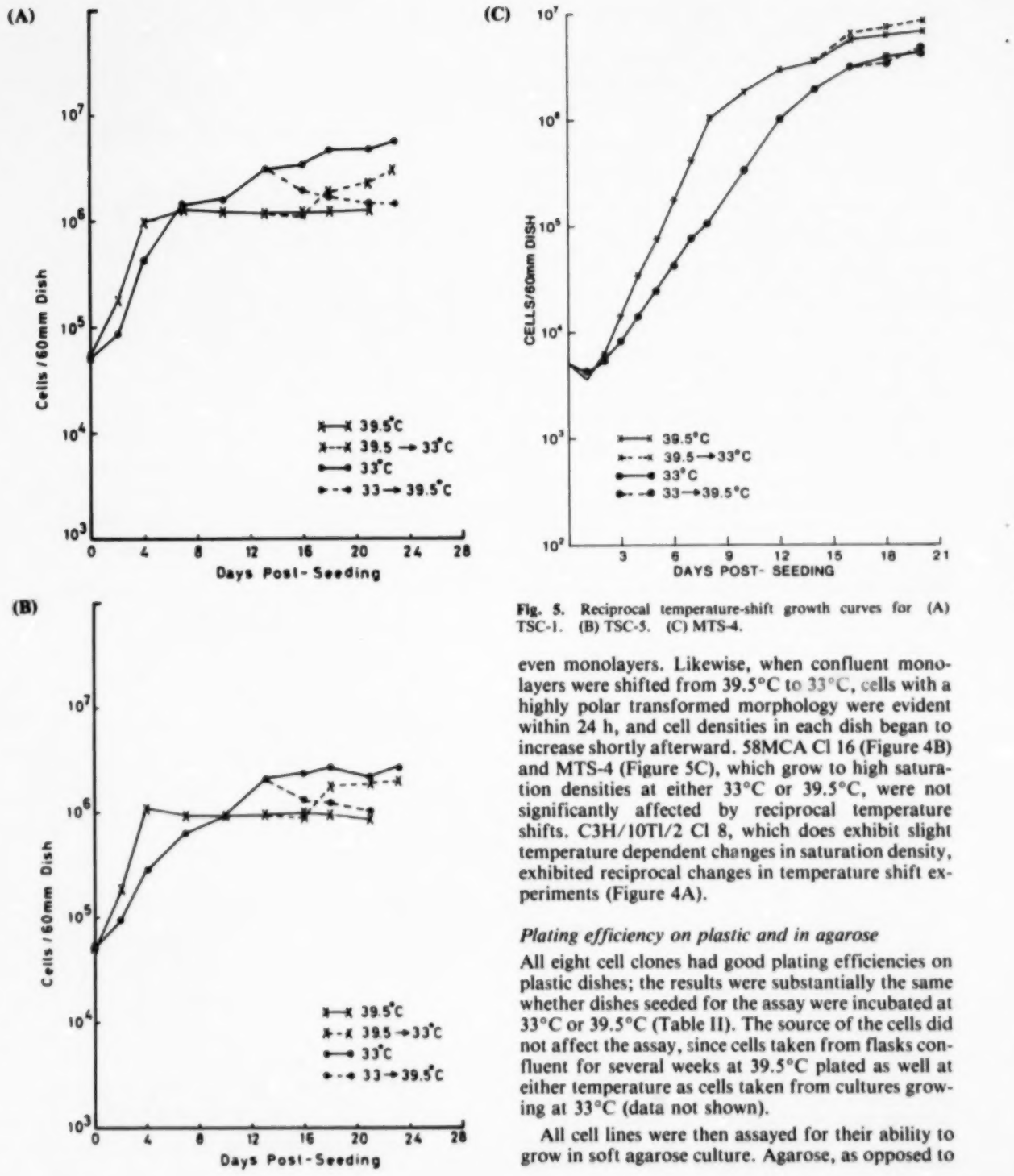

Fig. 5. Reciprocal temperature-shift growth curves for (A) TSC-1. (B) TSC-5. (C) MTS-4.

even monolayers. Likewise, when confluent monolayers were shifted from 39.5°C to 33°C, cells with a highly polar transformed morphology were evident within 24 h, and cell densities in each dish began to increase shortly afterward. 58MCA Cl 16 (Figure 4B) and MTS-4 (Figure 5C), which grow to high saturation densities at either 33°C or 39.5°C, were not significantly affected by reciprocal temperature shifts. C3H/10Tl/2 Cl 8, which does exhibit slight temperature dependent changes in saturation density, exhibited reciprocal changes in temperature shift experiments (Figure 4A).

Plating efficiency on plastic and in agarose

All eight cell clones had good plating efficiencies on plastic dishes; the results were substantially the same whether dishes seeded for the assay were incubated at 33°C or 39.5°C (Table II). The source of the cells did not affect the assay, since cells taken from flasks confluent for several weeks at 39.5°C plated as well at either temperature as cells taken from cultures growing at 33°C (data not shown).

All cell lines were then assayed for their ability to grow in soft agarose culture. Agarose, as opposed to

Table II

Effect of temperature on plating efficiency on plastic and in agarose.

Cell line	Plating efficiency on plastic (%)			Plating efficiency in agarose (%)		
	39.5°C	33°C	Ratio 33/39.5°C	39.5°C	33°C	33°C/39.5°C
C3H/10T1/2	20.3 ± 1.5	26.9 ± 2.0	1.33	0.001	0.001	---
58MCA Cl 16	41.4 ± 2.9	51.3 ± 0.5	1.24	31.6 ± 2.2	31.3 ± 3.8	0.99
TSC-1	32.9 ± 0.5	29.3 ± 5.4	1.12	0.002 ± 0.003	16.4 ± 6.1	8200
TSC-2	22.7 ± 6.0	25.5 ± 3.2	0.89	22.0 ± 5.6	20.2 ± 1.7	0.92
TSC-5	32.7 ± 0.6	26.6 ± 5.1	1.23	28.9 ± 5.7	32.8 ± 4.4	1.10
MTS-1	34.9 ± 3.1	30.7 ± 0.9	0.88	0.004 ± 0.002	22.8 ± 6.3	5700
MTS-4	28.5 ± 2.5	31.7 ± 3.0	1.11	4.8 ± 1.8	22.2 ± 0.7	5.0
MTS-6	33.4 ± 6.0	31.8 ± 1.6	0.95	0.5 ± 0.3	5.4 ± 0.6	10.8

All results are the mean of 3 – 5 experiments as described in **Materials and Methods.**

agar, was used in the assay since it generally results in higher plating efficiencies and larger colony sizes (29; Boreiko and Heidelberger, unpublished results). As shown in Table II, 58MCA Cl 16 cells grew well at either 33°C or 39.5°C, while C3H/10T1/2 Cl 8 cells were unable to form colonies at either temperature. All mutant lines grew in agarose at 33°C; the colonies formed ranged in size from several hundred to several thousand cells. TSC-2 and TSC-5 were equally capable of anchorage independent growth at 39.5°C, and formed colonies with a plating efficiency and size comparable to that observed at 33°C. MTS-4 and MTS-6 showed a decrease (about 90%) in their ability to form colonies at 39.5°C. The colonies that formed at 39.5°C were frequently smaller, but still usually consisted of several hundred cells. TSC-1 and MTS-1 grew with very poor plating efficiency at 39.5°C, the few colonies which were observed were very small and consisted usually of only 25 – 50 cells. The dramatic difference in the colony forming ability of TSC-1 is illustrated in Figure 3G-H.

Growth in low-serum medium

As is frequently observed in other cell systems (30), oncogenically transformed C3H/10T1/2 cells show reduced serum requirements for growth at 37°C (31,32). 58MCA Cl 16 cells are able to proliferate well in medium containing 1% FCS, while the growth of C3H/10T1/2 cells is inhibited (32). Therefore, it was of interest to examine the growth of all cell lines in 1% FCS at 39.5°C and 33°C (Table III).

58MCA Cl 16 cells grew well in medium containing 1% FCS at 39.5°C, while C3H/10T1/2 Cl 8 cells proliferated much more slowly. When the growth of the ts mutants was examined, only MTS-4 grew well in 1% FCS. The remaining five mutants did not display any capacity for growth after 14 days of incubation at 39.5°C in 1% FCS.

When the same experiments were performed in parallel at 33°C, the results were not nearly so clear. 58MCA Cl 16, MTS-4 and C3H/10T1/2 Cl 8 cells grew at very similar rates in 1% serum. The enhanced growth capacity of C3H/10T1/2 Cl 8 cells at 33°C was surprising and is of unknown significance. The remaining five mutant lines all grew at 33°C, although for some lines growth was very slow and irregular. Thus, five of six mutant lines showed ts differences in growth in 1% serum, but it would appear that the ts lesion exerts some effect on growth rate at both 33°C and 39.5°C.

Cell surface morphology

Efforts were made to determine whether the temperature at which cells were grown influenced their surface morphology as observed by scanning electron microscopy. Oncogenic transformation of C3H/10T1/2 cells is associated with the appearance of microvilli and blebs on the surface of non-mitotic cells (33); non-transformed cells generally appeared to have flat and smooth cell surfaces. While temperature-dependent differences were observed in the ts mutant lines, considerable heterogeneity was evident in many of the sample preparations. In addition, results with some of the cell lines examined were quite variable. Data collected in these studies are presented in Table III, which describes the average percent of cells possessing microvilli at 33°C or 39.5°C.

As expected, 58MCA Cl 16 cells grown at 39.5°C or 33°C possessed numerous microvilli and blebs on the cell surface. Cells growing at 33°C were not spread as well as those growing at 39.5°C, an indica-

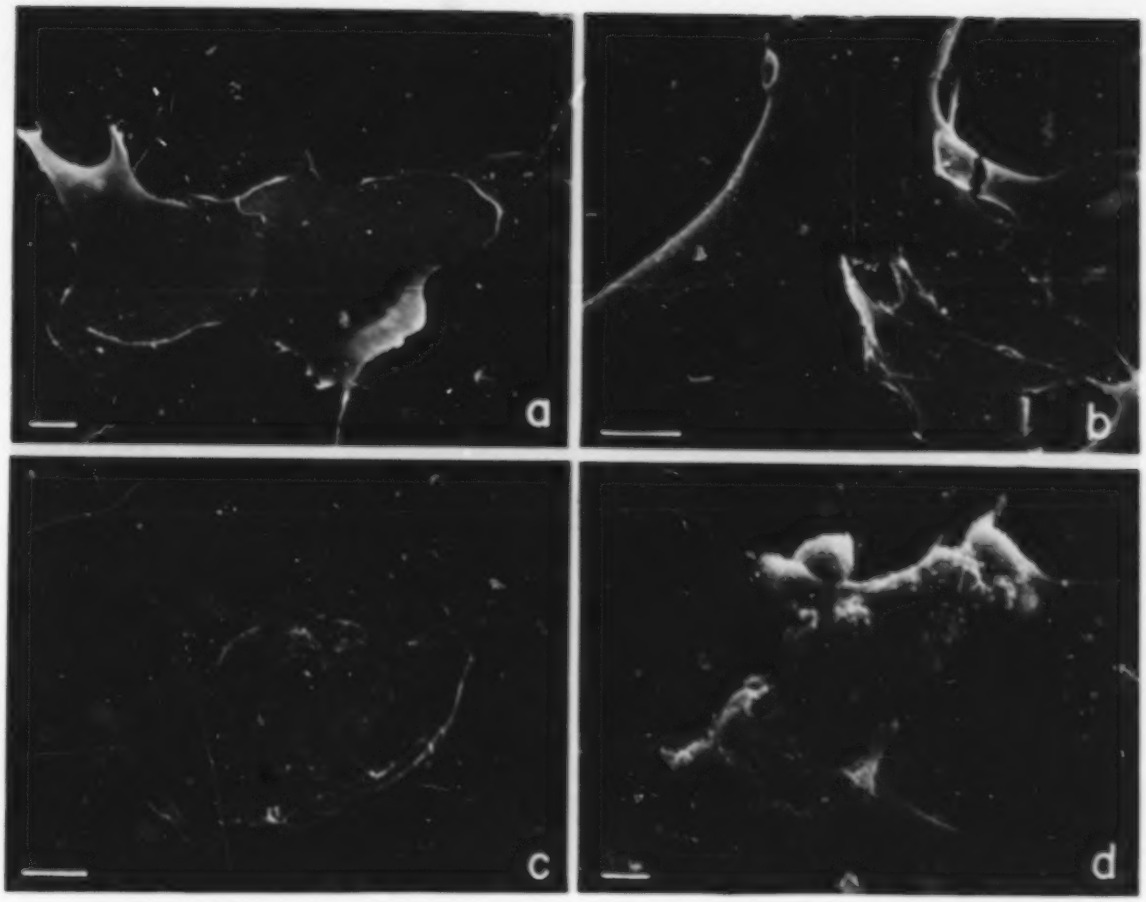

Fig. 6. Scanning electron micrographs of wild-type cells. Bar = 8 μM. (A) C3H/10T1/2 Cl 8 at 39.5°C. (B) C3H/10T1/2 Cl 8 at 33°C. (C) 58 MCA Cl 16 at 39.5°C. (D) 58 MCA Cl 16 at 39.5°C.

tion of the attachment problems noted earlier, leading to some difference in the overall appearance of cells growing at the two different temperatures (Figure 6A-B). C3H/10T1/2 Cl 8 cells were well spread at both temperatures and generally had smooth cell surfaces. Curiously, small numbers of stubby microvilli were present in some cells in occasional preparations of cells growing at 33°C (Figure 6C-D).

Results obtained with the ts mutants showed qualitative as well as quantitative differences. A high percentage of MTS-4 cells possess microvilli at both 39.5°C and 33°C. The number of microvilli per cell was increased at 33°C, however, and unusually active cell surfaces were seen (Figure 7C-D). The five remaining mutant clones showed definite temperature-dependent differences in the number of cells possessing microvilli. Both the number of cells with microvilli and number of microvilli per cell were higher at 33°C than at 39.5°C, as is shown for TSC-5 (Figure 7A-B). In occasional cell preparations, a high percentage of cells growing at 39.5°C possessed microvilli as well. This phenomenon was most often encountered with TSC-2, and data from these occasional anomalous experiments were not used in the compilation of Table III (see **Discussion**).

The effect of changes in temperature on cell surface morphology was examined with TSC-5. When cells growing at 39.5°C were shifted to 33°C, the percentage of cells possessing microvilli rose from 34% to 40% within 30 min after the shift and steadily increased until 90% of the cells possessed microvilli after 48 h. Similarly, cultures shifted from 33°C to 39.5°C showed rapid decreases in the number of cells possessing microvilli. Just 30 min after the shift, the percent of cells with microvilli declined from 89% to

Table III

Effect of temperature on growth in 1% FCS and cell surface morphology.

Cell line	Doubling time in 1% FCS (h)[a]		% of cells which possess microvilli[b]	
	39.5°C	33°C	39.5°C	33°C
C3H/10T1/2	68.1 ± 1.4	41.8 ± 1.1	7.0 ± 5.6	22.0 ± 11.0
58MCA Cl 16	31.1 ± 2.5	45.4 ± 0.9	84.0 ± 5.0	88.0 ± 2.0
TSC-1	NG	118 ± 29.0	11.0 ± 3.0	65.0 ± 8.0
TSC-2	NG	45.2 ± 4.0	29.0 ± 6.0[c]	90.0 ± 10.0
TSC-5	NG	63.4 ± 10.6	38.0 ± 9.0	96.0 ± 5.0
MTS-1	NG	96.2 ± 10.3	12.0 ± 3.0	90.0 ± 8.0
MTS-4	35.2 ± 6.4	43.5 ± 5.6	74.0 ± 14.0	95.0 ± 0.3
MTS-6	NG	85.8 ± 9.1	47.0 ± 6.0[c]	88.0 ± 3.0

[a] NG = no growth after 14 days of incubation. All values were determined in triplicate.

[b] Values are the average of 2 – 5 experiments for each cell line; 100 cells were observed per experiment.

[c] Much higher values were occasionally seen in some samples.

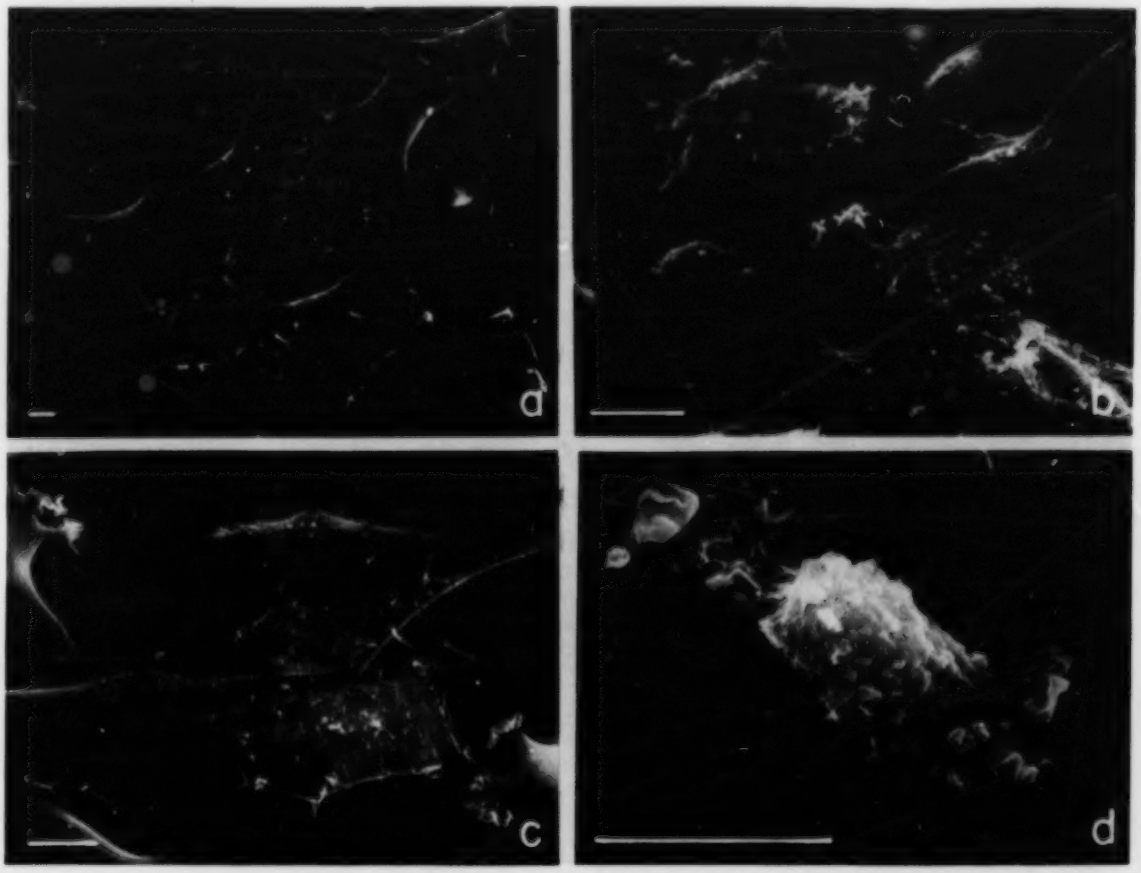

Fig. 7. Scanning electron micrographs of mutant cells. Bar = 8 μM. (A) TSC-5 at 39.5°C. (B) TSC-5 at 33°C. (C) MTS-4 at 39.5°C. (D)MTS-4 at 33°C.

71%, the decline continuing until only 30% of the cells possessed microvilli after 24 h.

Stability of the ts mutants

All ts mutants have been maintained in continuous culture at 33°C for at least 17 passages (about 6 months) without loss of ts effects on morphology, saturation density, or anchorage independence. In the absence of rigorous techniques to select for revertants, reversion frequencies were difficult to obtain. TSC-1, TSC-5 and MTS-6 grow to form stable confluent monolayers at 39.5°C. If confluent dishes were allowed to incubate for 3 weeks at 39.5°C with weekly renewal of the medium, a small percentage of the dishes developed foci of transformed cells similar to the oncogenic Type III foci, described by Reznikoff *et al.* (23), which appear in C3H/10T1/2 transformation assays. When such foci were picked with cloning rings and grown in mass cullture, they behaved as non-ts transformed cells (data not shown), and were thus considered to be revertants.

The frequency with which these revertants appeared was independent of the number of cells initially seeded. This suggests that revertants are not preexisting in stock cultures to any significant extent, but rather are generated with cell division. Since the expression time and cell division dependence for the reversion process have not been rigorously defined, only rough estimates of reversion frequencies could be made. Thus TSC-1, TSC-5 and MTS-6 appear to revert with frequencies less than 2×10^{-6}. The frequency of TSC-1 revertants is increased by treatment with MNNG in a dose-dependent fashion (data not shown). Estimates of reversion frequency were not obtained for TSC-2, MTS-1 or MTS-4. When held at confluence for 3–4 weeks at 39.5°C, dishes of MTS-1 and TSC-2 developed multiple foci. This may indicate high reversion rates, or may have been due to continuous generation of revertants with the extensive cell division induced by the weekly renewal of fresh medium. No means to determine MTS-4 reversion frequency were available.

Discussion

58MCA Cl 16, an oncogenic methylcholanthrene-transformed variant of C3H/10T1/2 mouse embryo cells, was mutagenized and subjected to multi-step procedures designed to enrich the cell population for the presence of mutants ts for expression of the transformed state. Each of the two procedures employed resulted in sufficient enrichment to permit the isolation of ts mutants by standard cloning techniques. Mutants designated "TSC" were isolated on the basis of their inability to continue growth in confluent cultures at 39.5°C. "MTS' mutants were isolated on the basis of an inability to grow while suspended in methyl cellulose at 39.5°C.

Three mutants were isolated from each enrichment protocol, each mutant being derived from independently mutagenized and enriched cultures of transformed cells. For all mutants, 33°C was selected as the temperature permissive for the expression of transformation and 39.5°C was non-permissive for the transformed state. All mutants were fully viable at either temperature, and are stable in long-term culture. At least three of the mutants appear to revert with low frequency.

The ts phenotypes of the mutant and parental cell lines are summarized in Table IV. As expected, all "TSC" mutants were ts for saturation density. All "MTS" mutants displayed ts effects on anchorage independent growth. 58MCA Cl 16 cells retained expression of transformed properties independent of the temperature of incubation. Likewise, C3H/10T1/2 Cl 8 cells generally behaved in a non-transformed fashion, although there were some

Table IV

Summary of cell phenotypes at 39.5°C and 33.0°C[a].

Cell line	Morphology	Saturation density	Anchorage independence	Growth in low serum	Cell surface
C3H/10T1/2	N	N	N	?	N
58MCA Cl 16	Tx	Tx	Tx	Tx	Tx
TSC-1	C	C	C	C	C
TSC-2	C	C	C	C	C
TSC-5	Tx	C	Tx	C	C
MTS-1	C	C	C	C	C
MTS-4	Tx	Tx	C	Tx	Tx
MTS-6	C	C	C	C	C

[a] N = non-transformed; Tx = transformed; C = conditional or ts behaviour; ? = ambiguous result.

curious anomalies. At 33°C, the growth of the cells in medium containing 1% serum was enhanced, and numerous short microvilli were occasionally seen on the cell surface. The significance of this behavior is not clear, although it may bear some relationship to the observation that a non-transformed mouse cell line acquired transformed characteristics when grown at low temperatures (15). In addition, short microvilli have been observed on the surface of non-oncogenic variants of C3H/10T1/2 (34) and thus may not be related to the process of oncogenesis.

In three of the mutant lines (TSC-1, MTS-1 and MTS-6), ts behavior was observed for all properties of transformation examined. In others, a ts phenotype was observed for some, but not all of the properties tested. TSC-2 was able to grow in agarose suspension culture at 39.5°C or 33°C, but was ts for the expression of all other properties of transformation. Similarly, TSC-5 behaved in a transformed fashion with respect to morphology and anchorage independence at both temperatures, but was ts for other properties. Finally, MTS-4 shows ts behavior for anchorage independence, but all other properties remained transformed at both permissive and non-permissive temperatures. Whether or not the slight ts behavior observed in this mutant is significant remains to be determined.

Caution should be exercised in interpreting data on both the ability of the mutants to grow in medium containing 1% serum and their cell surface morphology. The ts lesions in five mutants prevented growth in low serum at 39.5°C, but frequently permitted slow rates of proliferation at 33°C. Thus, while definite temperature-dependent effects on the growth of cells in 1% FCS were observed, growth was atypical of what one would expect for transformed or non-transformed C3H/10T1/2 cells. We have assumed that the differences observed in growth at the two temperatures reflect a shift in the neoplastic potential of the cells, but other explanations may be possible.

The opposite was true of studies on cell surface morphology. For several of the mutant lines, cells growing at 39.5°C frequently possessed numerous microvilli. Microvilli were generally much more apparent at 33°C, indicative of a temperature-dependent difference, but the difference was frequently not as great as one might have expected if a transition from a transformed to a non-transformed state had occurred. This may reflect leakiness of the ts lesion.

The diversity of phenotypes observed in our mutants has several interesting implications. First, it suggests that each mutant arose independently by virtue of separate mutagenic events. Second, the diversity demonstrates that while the expression of many transformed properties sometimes appears to be linked, this linkage is not obligatory. Traits such as anchorage independence, loss of density-dependent control of cell division, and transformed morphology were observed to be expressed independently of one another. This further leads us to suggest that there are multiple cellular elements that can modulate expression of the transformed state. Mutants with coordinate ts behavior for all properties may possess an alteration in a cellular component intimately involved in the expression of most, if not all, properties of the transformed state. Evidence for the existence of such a cellular component has recently been provided by Shih *et al.* (35) who found that the chemically transformed state can be transmitted from transformed to non-transformed C3H/10T1/2 cells by DNA transfection procedures. More than one component of this nature could exist. Mutants with partial ts behavior lead us to postulate the existence of additional non-overlapping cellular controls more proximal to the expression of individual traits or set of traits. Based on the precedent established through the study of ts mutants in other systems (10), the affected cellular components are probably proteins that have had their thermodynamic stability altered by a point mutation.

Dissociation of properties associated with oncogenic transformation is not, of course, without precedent. When Syrian hamster embryo cells are transformed by benzo[a]pyrene, the cells exhibit a progressive acquisition of transformed properties with passaging (36); the transformed morphology was observed prior to anchorage independence. Similarly, a non-selective analysis of SV40 transformed 3T3 cells showed that numerous partial transformants expressed some, but not all, properties of the transformed state (37). In addition, revertants of SV40 transformed cells are sometimes observed to revert for some, but not all transformed properties (27).

Some ts mutants of RNA tumor viruses (9,38) and cells transformed by AEV or MC29 (39) exhibit differential expression of some properties of transformation. In particular, cells infected by the ts mutants are frequently fully transformed at non-permissive temperatures with respect to growth properties, but show ts behavior with respect to various biochemical parameters, such as hexose transport and release of plasminogen activator. This partial ts behavior is suprising in view of the observations that the *src* sequence of these viruses appears to code for a single phosphoprotein (5). Although the dissociation of transformed properties observed in those viral mutants is not as extensive as that observed for ts

mutants of 58MCA Cl 16, the hypotheses offered to explain the behavior of ts viral mutants certainly must be considered.

Ts viral mutants displaying dissociation of transformed properties may, for example, be leaky mutants, the ts lesion only partially limiting the interaction of a transforming gene product with its primary cellular target (38). Transformed properties requiring full activity of the *src* gene product as it interacts with its primary target would be lost at the non-permissive temperature. Other properties of transformation, more sensitive to a *src* related change, could be retained. Evidence against this simple model for viral ts mutants has been obtained (40), lending support to other models that attempt to explain the "leaky" behavior of some ts mutants. For example, a ts lesion in *src* might differentially affect the interaction of the *src* gene product with multiple primary targets.

The possibility must be considered that our ts mutants are due to alterations in the same cellular component, phenotypic variability among mutants being caused by leakiness of the mutations. We believe the diversity of phenotypes observed here argues against leakiness. However, we cannot exclude the possibility that all our mutants are caused by lesions in the same gene product, and that secondary cellular changes prior or subsequent to introduction of the ts lesion might alter expression of the mutation.

The question of whether or not the diverse phenotypes of the mutants isolated here are due to alterations in different cellular components involved in the regulation of the transformed state might be answered by cell fusion techniques. Preliminary hybridization studies with ts mutants of spontaneously transformed cells suggest the existence of multiple complementation groups (41). Similar studies are in progress in our laboratory.

The mutants isolated here represent potentially powerful tools for studying numerous aspects of chemical oncogenesis in the C3H/10T1/2 system. The model systems they provide should greatly facilitate efforts to delineate cellular processes and products involved in the maintenance of the chemically transformed state. Combined with ongoing studies to investigate the tumorigenicity of our mutants, those *in vitro* transformation properties most relevant to chemical oncogenesis may be defined at the cellular and eventually at the molecular level.

Acknowledgements

We thank Ms Sharon Lynch for her help with cell cultures and Ms Ellen Morgan for the preparation of scanning electron micrographs. We also acknowledge the help of Mr J. Stephen Johnston in the preparation of graphic materials for this manuscript. This work was supported in part by Contract N01 CP 65831 from the National Cancer Institute, NIH, and by Grant R-80-5208 from the Environmental Protection Agency.

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